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Antifungal Activities of Tacrolimus and Azole Agents against the Eleven Currently Accepted *Malassezia* Species

Takashi Sugita,[1]* Mami Tajima,[3] Tomonobu Ito,[3] Masuyoshi Saito,[3] Ryoji Tsuboi,[3] and Akemi Nishikawa[2]

Department of Microbiology[1] and Department of Immunobiology,[2] Meiji Pharmaceutical University, Kiyose, Tokyo, Japan, and Department of Dermatology, Tokyo Medical University, Shinjuku, Tokyo, Japan[3]

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The lipophilic yeast *Malassezia* is an exacerbating factor in atopic dermatitis (AD) and colonizes the skin surface of patients with AD. With the goal of reducing the number of *Malassezia* cells, we investigated the antifungal activities of a therapeutic agent for AD, tacrolimus, and the azole agents itraconazole and ketoconazole against *Malassezia* species in vitro. We examined 125 strains of the 11 currently accepted *Malassezia* species by using the agar dilution method. All strains of the 11 *Malassezia* species were very susceptible to both azole agents, with MICs ranging from 0.016 to 0.25 μg/ml. Tacrolimus had antifungal activities against half of the strains, with MICs ranging from 16 to 32 μg/ml. Two of the major cutaneous floras, *Malassezia globosa* and *Malassezia restricta*, have several genotypes in the intergenic spacer region of the rRNA gene; the azole agents had slightly higher MICs for specific genotype strains of both microorganisms. A combination of azole agents and tacrolimus had a synergistic effect against *Malassezia* isolates, based on a fractional inhibitory index of 0.245 to 0.378. Our results provide the basis for testing these agents in future clinical trials to reduce the number of *Malassezia* cells colonizing the skin surface in patients with AD.

Although lipophilic yeasts, *Malassezia* spp., colonize the skin surface of healthy individuals, they may also cause seborrheic dermatitis (SD), pityriasis (tinea) versicolor, and *Malassezia* folliculitis and may exacerbate atopic dermatitis (AD) (1). AD is a common chronic inflammatory skin disease. The standard treatment of AD is topical corticosteroids and topical immunomodulating agents, although some patients do not respond to these treatments. Cutaneous microorganisms are considered an exacerbating factor. Although large numbers of lipophilic *Malassezia* species organisms colonize the skin surfaces of both AD patients and healthy subjects, anti-*Malassezia*-specific immunoglobulin E antibody is detected only in AD patient sera (14, 16, 32). This is probably owing to the disrupted barrier function of the skin surface and the effects of scratching on sensitization to the organisms (30). The application of topical antifungal agents to AD patients decreases *Malassezia* colonization and the severity of eczematous lesions (2), suggesting that *Malassezia* species play a role in atopic dermatitis. In addition, several candidate *Malassezia* antigens have been implicated in the pathogenesis of AD (15, 19, 20, 23, 34).

In 1996, the taxonomy of the genus *Malassezia* was revised by Guého et al. (8). The authors described seven species (*Malassezia furfur*, *M. globosa*, *M. obtusa*, *M. restricta*, *M. slooffiae*, *M. sympodialis*, and *M. pachydermatis*). Subsequently, Japanese researchers found another four new species: *Malassezia dermatis* (25), *M. yamatoensis* (28), *M. japonica* (27), and *M. nana* (11) were isolated from an AD patient, SD patients, a healthy individual, and an animal, respectively, between 2002 and 2004. At present, 11 species have been accepted in this genus. By use of the revised taxonomy, the correlation between cutaneous *Malassezia* floras and each skin disease has been investigated. Sugita et al. (24) identified the major *Malassezia* floras as *M. globosa* and *M. restricta* by using a PCR-based nonculture method. In addition, *M. globosa* and *M. restricta*

TABLE 1. *Malassezia* strains used for drug susceptibility testing

Species	Genotype	No. of strains	Location(s)	Source[a] (no.)
M. globosa	I	11	Japan	AD patients (11)
	II	4	Japan	AD patients (4)
	III	6	Japan	AD patients (4), HS (2)
	IV	6	Japan	HS (6)
	Total	27		
M. restricta	I	8	Japan, Brazil	AD patients (8)
	II	15	Japan	AD patients (5), HS (10)
	Total	23		
M. slooffiae		12	Japan	AD patients (12)
M. furfur		12	Japan	AD patients (12)
M. obtusa		9	Japan	AD patients (9)
M. nana		4	Japan, Brazil	Animal (4)
M. dermatis		3	Japan	AD patients (3)
M. japonica		2	Japan	HS (2)
M. yamatoensis		2	Japan	SD patients (2)
M. pachydermatis		6	Hungary	Animal (6)

[a] HS, healthy subject.

* Corresponding author. Mailing address: Department of Microbiology, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan. Phone: 81-424-95-8762. Fax: 81-424-95-8762. E-mail: sugita@my-pharm.ac.jp.

TABLE 2. Antifungal susceptibilities of *Malassezia* strains to itraconazole

Species	Cumulative % inhibited at the following MIC (μg/ml)[a]										
	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16
M. globosa											
Genotype:											
I	45.5 (5)	9.1 (1)	18.2 (2)	18.2 (2)	9.1 (1)						
II	100 (4)										
III	66.7 (4)	16.7 (1)	16.7 (1)								
IV	83.3 (5)	16.7 (1)									
AD patients	57.9 (11)	10.5 (2)	15.8 (3)	15.8 (3)							
Healthy subjects	87.5 (7)	12.5 (1)									
M. restricta											
Genotype:											
I	37.5 (3)	25 (2)	25 (2)	12.5 (1)							
II	73.3 (11)	20 (3)	6.7 (1)								
AD patients	38.5 (5)	30.8 (4)	23.1 (3)		7.7 (1)						
Healthy subjects	90 (9)	10 (1)									
M. sympodialis	76 (19)	8 (2)	8 (2)	4 (1)							
M. slooffiae	83.3 (10)	16.7 (2)									
M. furfur	66.7 (8)	25 (3)		8.3 (1)							
M. obtusa	88.9 (7)	11.1 (1)									
M. nana	100 (4)										
M. dermatis	100 (3)										
M. japonica	100 (2)										
M. yamatoensis	100 (2)										
M. pachydermatis	100 (6)										

[a] Numbers of strains examined are shown in parentheses.

consisted of four and two strains with different genotypes, respectively (26, 29). In the former species, two of the four genotypes were isolates from AD patients, one was from healthy subjects, and the remaining genotype included strains from both AD patients and healthy subjects. In the latter species, one genotype was an isolate from a healthy subject, and the other included isolates from both AD patients and healthy subjects.

In this study, we investigated three items: the in vitro susceptibilities of all 11 currently accepted *Malassezia* species to an immunomodulating agent (tacrolimus) and two antifungal agents (itraconazole [ITC] and ketoconazole [KTZ]), their in vitro susceptibilities to a combination of tacrolimus and an azole agent, and the in vitro susceptibilities of the strains of *M. globosa* and *M. restricta* with each genotype to these three agents.

MATERIALS AND METHODS

***Malassezia* isolates.** We examined 125 strains of 11 *Malassezia* species for their in vitro drug susceptibilities to tacrolimus and azole agents (ITC and KTZ), as shown in Table 1. The *Malassezia* strains were isolated mainly from AD outpatients and healthy volunteers. Animal isolates of *M. nana* and *M. pachydermatis* were provided by R. Kano of Nihon University and K. Takeo of Chiba University, respectively. OpSite transparent dressings (3 by 7 cm; Smith and Nephew Medical Ltd., Hull, United Kingdom) were applied to the scalp, back, arm, and nape of the neck of each subject. The samples were then transferred onto modified Leeming and Notman agar (mLNA) (10 g glucose, 10 g peptone, 8 g bile salts [OXOID, Hampshire, United Kingdom], 2 g yeast extract, 0.5 g glycerol monostearate, 15 g agar, 10 ml glycerol, 5 ml Tween 60, and 20 ml olive oil) containing 50 μg of chloramphenicol (Sankyo, Tokyo, Japan) and incubated at 32°C until yeast colonies were recovered. All 125 *Malassezia* isolates were identified by using rRNA gene sequence analysis. The isolated microorganisms were maintained on mLNA medium at 32°C.

Drugs. ITC and KTZ were kindly supplied by Janssen Pharmaceutical Company (Tokyo, Japan) and were diluted in dimethyl sulfoxide (Wako Chemical, Osaka, Japan). Stock solution was stored at −20°C until use. The injectable tacrolimus solution was purchased from Fujisawa Pharmaceutical Company (Osaka, Japan).

Drug susceptibility testing. In vitro drug susceptibility was determined according to the method of Gupta et al. (9), with slight modification. Briefly, the drugs were diluted in 200 μl of mLNA broth, to make a dilution series with doubled concentrations ranging from 0.16 to 320 μg/ml. To each diluted drug concentration, 1,800 μl of melted mLNA medium was added, resulting in final concentrations ranging from 0.016 to 32 μg/ml. The surface of each agar plate was inoculated with 50 μl of cell suspension and incubated for 7 days at 32°C. The cell growth was compared with the growth in a drug-free control, according to the following scale: 0, no visible yeast colonies on the agar medium; 1+, 25% growth in comparison with control; 2+, 50% of control growth; 3+, 75% of control growth; and 4+, growth similar to that of the control (9). MIC testing was carried out at least three times.

Synergy testing. The interactions of tacrolimus and the azole agents were estimated by antimicrobial susceptibility testing on mLNA agar medium, to test for synergy between these agents. The fractional inhibitory index (FIX) was calculated from the fractional inhibitory concentrations (FIC) as follows: FIX = FIC(ITC or KTZ) + FIC(tacrolimus), where FIC(ITC or KTZ) = [MIC(ITC and KTZ in combination)]/[MIC(ITC) + MIC(KTZ)] and where FIC(tacrolimus) = [MIC(tacrolimus in combination)]/[MIC(tacrolimus alone)]. The results were interpreted as follows: <0.5, synergy, and 0.5 to 4, indifferent (3).

TABLE 3. Antifungal susceptibilities of *Malassezia* strains to ketoconazole

Species	Cumulative % inhibited at the following MIC (μg/ml)[a]										
	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16
M. globosa											
Genotype											
I	45.5 (5)	9.1 (1)	18.2 (2)	27.3 (3)							
II	100 (4)										
III	66.7 (4)	16.7 (1)	16.7 (1)								
IV	83.3 (5)	16.7 (1)									
AD patients	57.9 (11)	10.5 (2)	15.8 (3)	15.8 (3)							
Healthy subjects	87.5 (7)	12.5 (1)									
M. restricta											
Genotype											
I	37.5 (3)	25 (2)	25 (2)	12.5 (1)							
II	80 (12)	13.3 (2)	6.7 (1)								
AD patients	46.2 (6)	23.1 (3)	23.1 (3)	7.7 (1)							
Healthy subjects	90 (9)	10 (1)									
M. sympodialis	76 (19)	12 (3)	4 (1)	4 (1)							
M. slooffiae	83.3 (10)	16.7 (2)									
M. furfur	66.7 (8)	16.7 (2)	8.3 (1)	8.3 (1)							
M. obtusa	88.9 (7)	11.1 (1)									
M. nana	100 (4)										
M. dermatis	100 (3)										
M. japonica	100 (2)										
M. yamatoensis	100 (2)										
M. pachydermatis	100 (6)										

[a] Numbers of strains examined are shown in parentheses.

RESULTS

In vitro susceptibility to tacrolimus and azole agents. The MICs of the three drugs are shown in Tables 2, 3, and 4. All the *Malassezia* species were very susceptible to both ITC and KTZ, with MICs ranging from 0.016 to 0.25 μg/ml, and approximately 80% of the strains had an MIC of ≤0.03 μg/ml. Tacrolimus had an antifungal effect against approximately 50% of the *Malassezia* strains, with MICs ranging from 16 to 32 μg/ml. This agent did not have an antifungal effect against the remaining 50% of the strains. In vitro susceptibility testing using a combination of the azole agents and tacrolimus was conducted using the six isolates of *M. furfur*, *M. globosa*, *M. restricta*, and *M. sympodialis* that had an MIC of ITC or KTZ of >0.125 μg/ml. When ITC or KTZ was combined with tacrolimus, the MICs against these isolates were reduced (Tables 5 and 6). The FIX of all these isolates were below 0.5 (synergistic effect).

In vitro susceptibilities of the strains of *M. globosa* and *M. restricta* with each genotype. Previously, we demonstrated that *M. globosa* and *M. restricta* organisms colonizing the skin surface of AD patients and healthy individuals were divided into four and two genotypes, respectively, by using the intergenic spacer region of the rRNA gene (Fig. 1 and 2; Table 1). For *M. globosa*, genotypes I and II are strains isolated from AD patients, genotype III contains strains obtained from both AD patients and healthy subjects, and genotype IV consists of strains isolated from healthy individuals only. The MICs of ITC and KTZ for this microorganism ranged from 0.016 to 0.25 μg/ml and from 0.016 to 0.125 μg/ml, respectively (Tables 2 and 3). All the strains with MICs of ITC and KTZ greater than 0.125 μg/ml belonged to genotype I. The MICs of ITC and KTZ for the genotype I strains were higher than those for the other genotype strains. For the tacrolimus MIC, no remarkable differences were found between the genotype strains (Table 4). For *M. restricta*, genotype I includes only strains isolated from AD patients, while genotype II includes strains obtained from both AD patients and healthy individuals. The MICs of ITC and KTZ for genotype I strains were higher than those for genotype II strains (Tables 2 and 3). For the tacrolimus MIC, no remarkable difference between the strains of each genotype was found (Table 4).

DISCUSSION

This study describes in vitro susceptibility testing of the 11 currently recognized *Malassezia* species to ITC, KTZ, and tacrolimus, and combined azole agent and tacrolimus. All 11 *Malassezia* species were very susceptible to both ITC and KTZ. These results are consistent with those documented in the literature (7, 9, 22). Within the very-susceptible range, how-

TABLE 4. Antifungal susceptibilities of *Malassezia* strains to tacrolimus

Species	Cumulative % inhibited at the following MIC (μg/ml)[a]										
	0.06	0.125	0.25	0.5	1	2	4	8	16	32	>32
M. globosa											
Genotype											
I									36.4 (4)	9.1 (1)	54.5 (6)
II									25 (1)	25 (1)	50 (2)
III										50 (3)	50 (3)
IV									33.3 (2)	33.3 (2)	33.3 (2)
AD patients									26.3 (5)	26.3 (5)	47.4 (9)
Healthy subjects									25 (2)	37.5 (3)	37.5 (3)
M. restricta											
Genotype											
I										25 (2)	75 (6)
II									26.7 (4)	20 (3)	53.3 (8)
AD patients									15.4 (2)	46.2 (6)	38.5 (5)
Healthy subjects									20 (2)	30 (3)	50 (5)
M. sympodialis									24 (6)	32 (8)	44 (11)
M. slooffiae									16.7 (2)	16.7 (2)	66.7 (8)
M. furfur									16.7 (2)	16.7 (2)	66.7 (8)
M. obtusa									11.1 (1)	33.3 (3)	55.5 (5)
M. nana										25 (1)	75 (3)
M. dermatis										33.3 (1)	66.7 (2)
M. japonica										100 (2)	
M. yamatoensis										50 (1)	50 (1)
M. pachydermatis										60 (4)	40 (2)

[a] Numbers of strains examined are shown in parentheses.

ever, variations in the susceptibilities of the major cutaneous floras *M. globosa* and *M. restricta* and the minor floras *M. sympodialis* and *M. furfur* to both agents was observed, with MICs ranging from 0.016 to 0.25 μg/ml. While the MIC of voriconazole for *Malassezia* species is similar to that of ITC and KTZ, that of fluconazole is greater than that of ITC and KTZ (9). In contrast to the azole agents, the variation in susceptibility to terbinafine is greater than that for the azole agents. Gupta et al. (9) examined 31 strains of *M. globosa*, *M. restricta*, and *M. furfur* and observed MICs of terbinafine ranging from 0.06 to 16.0, 0.06 to 4.0, and <0.03 to 32.0 μg/ml, respectively. We found that the susceptibilities of genotypes of *M. globosa* and *M. restricta* to ITC and KTZ were correlated. Although a limited number of strains was examined, genotype I strains, which were obtained from AD patients only, had higher MICs for ITC and KTZ than did the strains with other genotypes. The reason for the correlation between genotype and susceptibility to ITC and KTZ is unclear. If an AD patient is given antifungal drugs repeatedly, the drug susceptibility of the fungi colonizing the patient's skin will change, but as no patient in this study received antifungal therapy, this possibility can be excluded. The cutaneous lipid composition in AD patients is slightly different from that of healthy subjects (10, 33). Such differences in composition may affect colonization by strains

TABLE 5. In vitro synergism between tacrolimus and ketoconazole

Species	Strain no.	KTZ			Tacrolimus			FIX
		MIC (μg/mL)		FIC index	MIC (μg/mL)		FIC index	
		KTZ alone	KTZ combined with tacrolimus		Tacrolimus alone	Tacrolimus combined with KTZ		
M. globosa	5	0.125	0.03	0.25	>32	8	0.125	0.375
	7	0.125	0.03	0.25	>32	8	0.125	0.375
	9	0.125	0.03	0.25	>32	4	0.125	0.375
M. restricta	6	0.125	0.03	0.25	>32	8	0.125	0.375
M. sympodialis	24	0.125	0.03	0.25	32	4	0.125	0.375
M. furfur	7	0.125	0.03	0.25	32	4	0.125	0.375

TABLE 6. In vitro synergism between tacrolimus and itraconazole

Species	Strain no.	ITC			Tacrolimus			FIX
		MIC (μg/ml)		FIC index	MIC (μg/ml)		FIC index	
		ITC alone	ITC combined with tacrolimus		Tacrolimus alone	Tacrolimus combined with ITC		
M. globosa	5	0.125	0.03	0.25	>32	8	0.125	0.375
	7	0.25	0.03	0.12	>32	8	0.125	0.245
	9	0.125	0.016	0.13	>32	16	0.25	0.378
M. restricta	6	0.25	0.03	0.12	>32	8	0.125	0.245
M. sympodialis	24	0.125	0.03	0.25	32	4	0.25	0.375
M. furfur	7	0.125	0.016	0.13	32	8	0.25	0.378

with different lipid requirements. In addition, the base ingredients in topical ointments affect the growth of *Malassezia* species (13). Of course, these factors do not affect drug susceptibility directly, but they do affect the selective colonization of microorganisms and might have an incidental effect that results in differences in drug susceptibility.

Clinical trials using ITC and KTZ in AD treatment have been conducted, and several studies have shown that these drugs are clinically effective in treating AD. AD patients with a positive radioallergosorbent test for *Malassezia*, who were treated with oral KTZ (200 mg/day for 2 months and 200 mg twice a week for another 3 months), had improved clinical scores for AD severity, particularly for the head and neck area (18). Oral ITC also improved the AD severity in patients with positive intradermal reactions to *Malassezia* and reduced the *Malassezia* radioallergosorbent test value (18). These investigations imply that ITC and KTZ therapies offer a promising treatment option for AD patients who are refractory to usual treatments. However, the optimal dosing regimens and treatment duration in larger clinical trials should be determined.

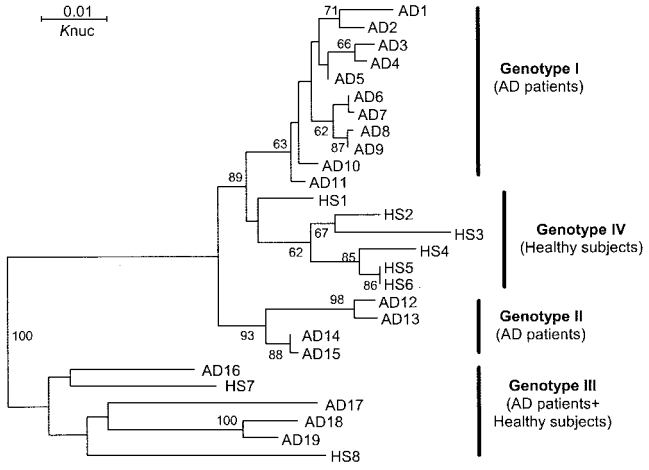

FIG. 1. Molecular phylogenetic tree of the *M. globosa* isolates. A tree was constructed from IGS1 sequences of the rRNA gene by using a neighbor-joining analysis (21) after the sequences were aligned by using ClustalW (31). The distances between sequences were calculated by using Kimura's two-parameter model (12). The numbers indicate the confidence levels from 100 replicate bootstrap samplings (frequencies less than 50% are not shown) (6). HS, healthy subject. *K*nuc, Kimura's parameter (12).

Tacrolimus, a therapeutic agent for AD treatment, also has an antifungal effect against approximately half of the *Malassezia* strains. The immunosuppressive drugs cyclosporine and tacrolimus target calcineurin, and these agents are toxic to *Candida albicans* and *Cryptococcus neoformans* (4). In addition, we demonstrated that tacrolimus, with either ITC or KTZ, has synergistic activity against *Malassezia*. These observations follow earlier reports on a combination of tacrolimus and fluconazole against *C. albicans* and *C. neoformans* strains. As immunosuppressive agents cannot be given to patients with deep-seated mycosis (immunocompromised hosts), the nonimmunosuppressive analog L-685,818 has been synthesized (5). The combination of topical tacrolimus and an azole agent can simultaneously treat AD and reduce the number of *Malassezia* cells colonizing the skin surface that are an exacerbating factor. While the synergistic mechanism of the combination of tacrolimus and azole agents is not known, Maesaki et al. (17) demonstrated that tacrolimus increases the intracellular concentration of the azole agent in their study of *C. albicans*. We found no ITC- or KTZ-resistant *Malassezia* strains. When azole-resistant *Malassezia* strains colonize the skin, combined treatment with tacrolimus can render them susceptible to azole agents.

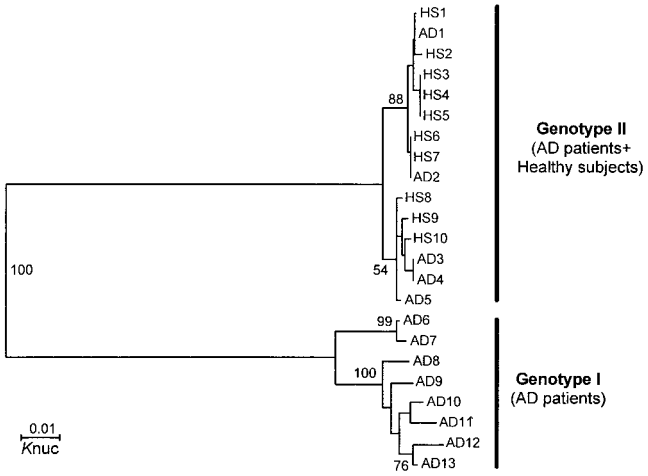

FIG. 2. Molecular phylogenetic tree of *M. restricta* isolates. The tree was constructed using the method described in the legend for Fig. 1. HS, healthy subject. *K*nuc, Kimura's parameter (12).

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